DESCRIPTION

METHODS AND COMPOSITIONS CONCERNING ALTERED YELLOW FEVER VIRUS STRAINS

BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Application No. 60/397,440 filed on July 19, 2002, which is hereby incorporated by reference in its entirety.

1. Field of the Invention

The present invention relates generally to the fields of molecular biology and virology. More particularly, it concerns nucleic acid compositions and methods for using such compositions to develop Yellow Fever vaccines.

2. Description of Related Art

The disease Yellow Fever, caused by a member of the *Flaviviridae* family designated Yellow Fever (YF) virus, is prevented by the use of live attenuated vaccine known as 17D. The 17D virus was developed by passage of Yellow Fever virus wild-type strain Asibi (isolated in Ghana in 1927) in chicken tissue. The 17D vaccine is manufactured by six producers worldwide who jointly manufacture approximately 100-150 million doses annually. The 17D vaccine has an excellent safety record with only 21 reports of expression of a neurovirulent phenotype. In recent years there have been three separate reports (in Brazil, Australia and USA) of 17D expression of a viscerotropic phenotype, with cases of apparent Yellow Fever-type disease, causing concern and is threatening the use of 17D vaccine.

Tesh *et al.* reported studies on three YF strains in hamsters (Tesh *et al.*, 2001). Two strains became viscerotropic only following intraperitoneal inoculation of virus and multiple liver-to-liver passages in hamsters. One strain, Jimenez, (isolated in Panama in 1974 from a human case) was unusual in that it caused viscerotropic disease in hamsters without adaptation by passage in hamsters and killed a proportion of animals.

There exists a clear need for vaccines that will stimulate an immune response in a subject, while reducing the potential for expression of a virulent phenotype. Thus, methods and compositions useful for the production and use of improved vaccines would be beneficial.

SUMMARY OF THE INVENTION

Compositions and methods of the present invention include provisions for the improvement of *flavivirus* vaccines so that the risk of disease is reduced or eliminated. In certain embodiments the *flavivirus* is a Yellow Fever virus. In other embodiments, a virus may be an altered 17D, 17D-204, 17DD, or other Yellow Fever vaccines. In various other embodiments the vaccine may be a chimeric vaccine, as described herein. Chimeric refers to a viral genome, viral polypeptide or viral particle that contains a discernable portion(s) of at least two viruses or virus strains, and may also include portions of non-viral nucleic acids and/or polypeptides.

In various embodiments, an isolated nucleic acid encoding a Yellow Fever virus with a viral genome that will require alteration of at least two nucleotides of a codon to convert the codon to a codon encoding any 1, 2, 3, 4, 5, 6 or 7 of the following amino acid alterations or any combination thereof is contemplated. The amino acid alterations to discourage include, but are not limited to a) an alteration to an envelope protein (described below) with a histidine at amino acid 27; b) an alteration to an envelope protein with a glycine at amino acid 28; c) an alteration to an envelope protein with an alanine at amino acid 155; d) an alteration to an envelope protein with an arginine at amino acid 323; e) an alteration to an envelope protein with an arginine at amino acid 331; f) an alteration to a NS2A protein (described below) with an alanine at amino acid 48; or g) an alteration to a NS4B protein (described below) with an isoleucine at amino acid 98. Each of the nucleic acid alterations may be used in combination with each and every other combination of the remaining nucleic acid alterations and/or other nucleic acid alteration in a 5' or 3' noncoding region (NCR) and/or nucleic acids encoding a core (C), a PrM, an M, an envelope (E), a NS1, a NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 protein(s) and combinations thereof. In a preferred embodiment, the amino acid alteration to discourage is one resulting in an envelope protein with a glycine at amino acid 28.

Also contemplated are methods of vaccination, viral production and screening for compounds that modulate viralinteraction or activity utilizing such a virus as described herein.

In various embodiments an isolated nucleic acid encoding a Yellow Fever virus with a viral genome that may include at least one of the following alterations: a) an alteration in the nucleic acid sequence resulting in an envelope protein (described below) with a histidine at amino acid 27; b) an alteration in the nucleic acid sequence resulting in an envelope protein with a glycine at amino acid 28; c) an alteration in the nucleic acid sequence resulting in an envelope protein with an alanine at amino acid 155; d) an alteration in the nucleic acid sequence resulting in an envelope protein with an arginine at amino acid 323; e) an alteration in the nucleic acid sequence resulting in an envelope protein with an arginine at amino acid 331; f) an alteration in the nucleic acid sequence resulting in a NS2A protein (described below) with an alanine at amino acid 48; or g) an alteration in the nucleic acid sequence resulting in a NS4B protein (described below) with an isoleucine at amino acid 98. Each of the alterations may be used in combination with each and every other combination of the remaining alterations and/or other alteration in a 5' or 3' noncoding region (NCR) and/or a core (C), a PrM, an M, an envelope (E), a NS1, a NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 protein(s) and combinations thereof, each of which is described below.

A nucleic acid sequence representative of a hamster passage 7 Yellow Fever virus sequence is presented in SEQ ID NO:1. A polypeptide sequence representative of a hamster passage 7 Yellow Fever virus sequence is presented in SEQ ID NO:2. SEQ ID NO:3 is a portion of SEQ ID NO:1 that encodes an envelope protein and SEQ ID NO:4 sets forth a polypeptide that represents a processed envelop protein. The location of all other protein may be determined by analysis of the genbank sequences described below.

The nucleic acids of the invention may be RNA or DNA. In some embodiments where the nucleic acid is DNA transcription will be oriented so that an infectious RNA will typically be transcribed from the DNA.

In various other embodiments, a nucleic acid encoding all or part of a viral genome may include at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more alterations. Alterations may be innocuous or render the virus more or less immunogenic, replication competent, virulent or alter other characteristics of the

virus. In certain embodiments, the nucleic acid the polynucleotide has a nucleic acid sequence as set forth in SEQ ID NO:1.

In other embodiments a nucleic acid comprising 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9,000, 10,000 or more and values there between of contiguous nucleotides of SEQ ID NO:1.

In yet other embodiments, a vaccine composition may include a Yellow Fever virus with a viral genome that includes at least one of the following alterations and may include any combination thereof: a) an alteration in the nucleic acid sequence encoding amino acid 323 of an envelope protein, wherein the first alteration requires more than one nucleotide change to encode an arginine; b) an alteration in the nucleic acid sequence encoding amino acid 27 of an envelope protein, wherein the second alteration requires more than one nucleotide change to encode a histidine; c) an alteration in the nucleic acid sequence encoding amino acid 28 of an envelope protein, wherein the second alteration requires more than one nucleotide change to encode a glycine; d) an alteration in the nucleic acid sequence encoding amino acid 155 of an envelope protein, wherein the second alteration requires more than one nucleotide change to encode an alanine; e) an alteration in the nucleic acid sequence encoding amino acid 331 of an envelope protein, wherein the second alteration requires more than one nucleotide change to encode an arginine; f) an alteration in the nucleic acid sequence encoding amino acid 48 of an NS2A protein, wherein the second alteration requires more than one nucleotide change to encode an alanine; or g) an alteration in the nucleic acid sequence encoding amino acid 98 of an NS4B protein, wherein the second alteration requires more than one nucleotide change to encode an isoleucine. The envelop protein is encoded by nucleotides 974 to 2452 of SEQ ID NO:1 and corresponds to amino acids 286 to 778 of SEQ ID NO:2.

The Yellow Fever virus viral genome may include at least two, three, four, five, six, or seven alterations in any combination. Typically, the vaccine composition is in a pharmaceutically acceptable formulation. Additionally, the vaccine composition may include the 17D virus, 17D-204 virus, 17DD virus, or other viral variants with any combination of alterations incorporated therein.

In still other embodiments, a method for producing an attenuated Yellow Fever virus including introducing into a Yellow Fever virus genome a missense mutation that would require two nucleotide changes to encode a supervirulence amino acid is contemplated. An attenuated virus refers to a virus that has been modified or treated to reduce or eliminate its ability to cause disease. A virus may be modified so as to yield a virus that has altered viremia and/or altered lethality consistent with changes discussed in the Examples.

In various embodiments, methods for producing a Yellow Fever virus vaccine may include: a) identifying a mutation that results in a missense mutation in a first Yellow Fever viral genome that is associated with an increased virulence of the virus; b) modifying an attenuated Yellow Fever viral genome by mutation of a codon associated with the missense mutation resulting in a reduced probability of reversion to a virulent phenotype. In certain embodiments, the method may include a missense mutation results in an envelope protein having an arginine at amino acid position 323 (SEQ ID NO:2) and may also include any combination of other alterations in the viral genome. The method may include modifying the attenuated Yellow Fever virus by substituting a second codon that encodes for a conservative amino acid change.

In other embodiments, a method for identifying a compound active against a viral infection including, but not limited to: a) providing a virus expressed from a viral construct comprising a nucleic acid encoding a Yellow Fever virus comprising an envelope protein with an arginine at amino acid 323; b) contacting said virus with a candidate substance; and c) comparing the infectious ability of the virus in the presence of said candidate substance with the infectious ability of the virus in a similar system in the absence of said candidate substance is contemplated. The method may also include a nucleic acid encoding a virus with an envelope protein including, but not limited to a histidine at amino acid 27, a glycine at amino acid 28, an alanine at amino acid 155, and/or an arginine at amino acid 331, as well as any other combination of alterations. In certain embodiments a nucleic acid sequence is that set forth in SEQ ID NO:1 or a polynucleotide sequence as set forth in SEQ ID NO:2, or other related flaviviral sequences.

In various embodiments, methods of vaccination including, but not limited to administering to a subject a Yellow Fever virus with a viral genome that includes at least one of the following alterations: a) an alteration in the nucleic acid sequence

encoding amino acid 323 of an/the envelope protein, wherein the first alteration requires more than one nucleotide change to encode an arginine; b) an alteration in the nucleic acid sequence encoding amino acid 27 of an/the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a histidine; c) an alteration in the nucleic acid sequence encoding amino acid 28 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a glycine; d) an alteration in the nucleic acid sequence encoding amino acid 155 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode an alanine; e) an alteration in the nucleic acid sequence encoding amino acid 331 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode an arginine; f) an alteration in the nucleic acid sequence encoding amino acid 48 of the NS2A protein, wherein the second alteration requires more than one nucleotide change to encode an alanine; or g) an alteration in the nucleic acid sequence encoding amino acid 98 of the NS4B protein, wherein the second alteration requires more than one nucleotide change to encode an isoleucine, as well as compositions used in vaccination are contemplated. The viral genome may also include at least a combination of two, three, four, five, six, seven or more alterations. The vaccine composition is typically in a pharmaceutically acceptable formulation. The vaccine composition may include, but not limited to having a 17D virus, 17D-204 virus, 17DD virus, or other Yellow Fever viral variants, as well as other viral strains and species. Methods of vaccination may include administration of an effective amount of a vaccine composition such that an immune response to virus is induced in a subject. In various embodiments, vaccination and vaccine compositions may include adjuvants and other excipients, as well as additional antigen(s) that may induce an immune response(es) to the same or other pathogen, foreign body, or organism.

Various embodiments of the invention may include, but are not limited to a) nucleic acid compositions comprising all or part of the nucleotide sequence of the hamster p7, viscerotropic Yellow Fever virus, as set forth in SEQ ID NO:1, or any other sequence incorporated herein by reference; b) methods of using a viscerotropic Yellow Fever virus nucleotide sequence for diagnosis of viscerotropic Yellow Fever strains by RT-PCR, gene probes, or expression of antigens c) methods of using the nucleotide sequence of a virulent Yellow Fever virus to identify molecular

determinants of viscerotropic disease, in particular using the Hamster as a model system; d) genetic engineering of molecular determinants of viscerotropic phenotypes to improve the safety of live attenuated Yellow Fever vaccines; and e) genetic engineering of the molecular determinants of a virulent phenotype in Yellow Fever virus similar or homologous nucleic acids or proteins in other virus that cause viral hemorrhagic fever. Molecular determinants may include, but are not limited to nucleic acids, polypeptides, complexes of polypeptides, and combinations of thereof. These may not be the same nucleotides/amino acids but could be the same or similar proteins. For example, information derived from Yellow Fever virus may be used to genetically alter dengue viruses, which may help in designing a dengue virus vaccine. It is contemplated that the present invention concerns nucleic acids that are viscerotropic and candidate vaccines that are engineered or selected to be non-viscerotropic or less viscerotropic, however, it will be generally understood that pathogenic viruses, and thus less pathogenic or non-pathogenic viruses are contemplated as well in the context of the invention. Therefore, compositions and methods involving viscerotropic viruses can be applied in the context of pathogenic viruses, as well as the generation of less-pathogenic vaccines. For example, attenuated viruses can be used in the context of the invention.

In other embodiments, various combinations of amino acid and nucleic acid alterations may be engineered into a *flavivirus*, *e.g.*, a Yellow Fever virus, in order to determine the characteristics of a virus containing various combinations of amino acid alterations. For example, an alteration in the nucleic acid sequence resulting in an envelope protein with a glycine at amino acid 28 may be studied in combination with an alteration in the nucleic acid sequence resulting in an envelope protein with a histidine at amino acid 27; an alteration in the nucleic acid sequence resulting in an envelope protein with an alanine at amino acid 155; an alteration in the nucleic acid sequence resulting in an envelope protein with an arginine at amino acid 323; an alteration in the nucleic acid sequence resulting in an envelope protein with an arginine at amino acid 331; an alteration in the nucleic acid sequence resulting in a NS2A protein with an alanine at amino acid 48; or an alteration in the nucleic acid sequence resulting in a NS4B protein with an isoleucine at amino acid 98. It is contemplated 2, 3, 4, 5, 6 or more alterations may be introduced and in a variety of combinations to determine the charateristics of a virus containg such. Each of the

alterations may be used in combination with each and every other combination of the remaining alterations and/or other alteration in a 5' or 3' noncoding region (NCR) and/or a core (C), a PrM, an M, an envelope (E), a NS1, a NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 protein(s) and combinations thereof, each of which is described below.

Information gained from the characterized viruses can then be used to screen for or generate candidate vaccines. Alteration(s) in amino acid(s) that results in a viscerotropic virus can be avoided in a candidate vaccine by engineering or selecting for a virus whose nucleic acid sequence at those positions renders it non-viscerotropic. In certain embodiments, the non-viscerotropic candidate vaccine virus contains a nucleic acid sequence that requires one or more nucleic acid alterations, such as at one or more positions of a codon, to generate a virus that has the amino acid sequence at that position as the viscerotropic virus. Resultant viruses can also be studied in animal systems to determine their applicability as candidate vaccine viruses. Thus, the present invention concerns screening and production methods for viruses to be used in vaccines.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

It is specifically contemplated that any limitation discussed with respect to one embodiment of the invention may apply to any other embodiment of the invention. Furthermore, any composition of the invention may be used in any method of the invention, and any method of the invention may be used to produce or to utilize any composition of the invention.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 illustrates an exemplary study of the survival of 3-4 week old Golden Syrian hamsters following inoculation with either parental Asibi p0 virus or viscerotropic Asibi p7 virus.

FIG. 2 illustrates an example of viremia in sub-adult hamsters inoculated with either Asibi p7 or Asibi p0 virus. Values shown are the average of 5-6 animals. Downward arrows indicate values that are at or below the limit of sensitivity for this assay.

FIG. 3A-3B shows exemplary H&E stained sections of hamster liver 6 days post infection (dpi). (FIG. 3A) Liver from mock-infected hamster. (FIG. 3B) Liver from hamster infected with Asibi/hamster p0. (FIG. 3C) Liver from hamster infected with Asibi/hamster p7.

FIG. 4A-4B illustrates exemplary liver pathology in hamsters inoculated with viscerotropic Asibi p7. Animal A was sacrificed on day 5 post infection due to severe illness. (Fig. 4A) Steatosis is expressed as a percentage of the total liver. (FIG. 4B) Hepatic necrosis and lobular inflammation are presented as a grade from 0-4 with 0 being none and 4 being severe. The remaining animals (FIG. 4B, animal B-E) were beginning to show signs of illness when they were sacrificed on day 6 post infection (pi).

FIG. 5A-5C illustrates exemplary H&E stained sections of hamster spleen 6 dpi. (FIG. 5A) Spleen from mock-infected hamster. (FIG. 5B) Splcen from hamster

infected with Asibi/hamster p0. (FIG. 5C) Spleen from hamster infected with Asibi/hamster p7.

FIG. 6 shows an example of the splenic abnormalities identified in 3-4 week old hamsters inoculated with Asibi p0 and Asibi p7 viruses.

FIG. 7 illustrates the three-dimensional structure of the YF virus E protein based on the crystallographic structure of TBE virus E protein (Rey *et al.*, 1995). The 5 amino acid positions that differ between the Asibi/hamster p0 and Asibi/hamster p7 E27, E28, E155, E323, and E331 are highlighted and labeled.

FIG. 8A-B shows a comparison between the viremia (A)and mortality (B) rates between Asibi hamster p6 and p7.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Compositions and methods of the present invention include provisions for the improvement of *flavivirus* vaccines so that the risk of disease is reduced or eliminated. In certain embodiments the *flavivirus* is a Yellow Fever virus. In other embodiments, a virus may be an altered 17D, 17D-204, 17DD, or other Yellow Fever vaccines. In various other embodiments the vaccine may be a chimeric vaccine, as described herein. Chimeric refers to a viral genome, viral polypeptide or viral particle that contains a discernable portion(s) of at least two viruses or virus strains, and may also include portions of non-viral nucleic acids and/or polypeptides.

In certain embodiments, viral variants are typically selected that demonstrate an increased virulence in a model host (*e.g.*, a hamster), a so-called supervirulent virus. Supervirulent refers to an organism or virus that demonstrates an increased or enhanced ability to cause injury or disease in a host organism, tissue, and/or cell. Viral isolates may be sequenced to identify nucleotide and/or amino acid changes associated with increased virulence. The information provided by the alterations associated with increased virulence may be used to genetically engineer mutations in other viruses either individually or in various combinations to improve the safety profile of an attenuated virus used as a vaccine. Thus, an engineered virus may then be used as a vaccine with a lower probability of reversion to a virulent phenotype. These alterations will reduce the probability of a reversion in the vaccine by increasing the number of mutational events necessary to alter an encoded amino acid to an amino acid associated with supervirulence or a virulent phenotype.

The information provided by the analysis of nucleotide sequences involved in viscerotropic disease will typically identify nucleotides and amino acids that should not be incorporated in any live attenuated Yellow Fever vaccine and in particular any equivalent position in other *flavivirus* vaccine. An equivalent position may be identified by homology or similarity to Yellow Fever virus sequences or similarity to motifs or conserved sequence characteristics between a Yellow Fever virus and other members of the *flavivirus* genus.

In an exemplary embodiment, the inventors have passaged a wild-type strain Asibi (Hahn et al., 1987; the parent strain of vaccine strain 17D) seven times in hamsters by liver-to-liver passage and have generated a variant of Asibi virus that is viscerotropic in hamsters, as well as demonstrating a virulent phenotype. Non-hamster passaged Asibi virus does not kill hamsters while Asibi hamster passage 7 virus kills hamsters (supervirulent phenotype).

The genome of Asibi hamster passage 7 (p7) virus has been sequenced and the nucleotide sequence changes associated with the hamster viscerotropic phenotype have been identified by comparing the genomes of non-hamster passaged Asibi virus and Asibi hamster p7 virus. There are a number of nucleotide and amino acid differences between the two viruses. In various embodiments of the invention these mutations may be used to improve 17D, other Yellow Fever virus vaccines, or other *flavivirus* vaccines.

In other embodiments of the invention, genetic engineering may be used to genetically manipulate the single-stranded, positive-sense RNA genome of Yellow Fever virus or other members of the *Flaviviridae* family. Genetic manipulation may introduce mutations into the 17D vaccine virus genome or the genome of another *flavivirus* vaccine virus to further attenuate the virus and reduce the viscerotropic disease potential of 17D. Infectious clones of strain 17D have been developed as a basis for chimeric vaccine (ChimeriVax™) platform to make chimeric 17D viruses containing the foreign envelope protein genes of other *flavivirus*. (*e.g.*, dengue, West Nile and Japanese encephalitis) (Acambis Inc., Cambridge MA). For example see U.S. Patent No. 6,184,024, which is incorporated herein by reference.

In general, the information on the molecular determinants of viscerotropism of Yellow Fever virus is sparse and there is little information regarding the molecular determinants involved in this or other hemorrhagic fevers resulting from *flavivirus*

infections. Embodiments of the invention will aid in the identification of these molecular mechanisms and provide for the engineering of improved vaccines.

In certain embodiments, the genomic nucleic acid sequence of Asibi hamster passage 7 virus, as compared with non-hamster passaged Asibi virus (Hahn *et al.*, 1987, which is incorporated herein by reference), may be used to identify molecular determinants of hamster viscerotropism.

I. *FLAVIVIRUS*

The genus *Flavivirus* is a member of the *Flaviviridae* family and includes the viral subgroups of Yellow Fever virus group, Tick-borne encephalitis virus group, Rio Bravo Group, Japanese encephalitis Group, Tyuleniy Group, Ntaya Group, Uganda S Group, Dengue Group, and Modoc Group. Members of the *Flavivirus* genus may produce a wide variety of disease states, such as fever, arthralgia, rash, hemorrhagic fever, and/or encephalitis. The outcome of infection is influenced by both the virus and host-specific factors, such as age, sex, genetic susceptibility, and/or pre-exposure to the same or a related agent. Some of the various diseases associated with members of the genus *Flavivirus* are Yellow Fever; Dengue Fever; and West Nile, Japanese, and St. Louis Encephalitides.

Virions of the *Flaviviridae* generally contain one molecule of a linear positive-sense single stranded RNA genome of approximately 10,000-11,000 nucleotides that replicates in the cytoplasm of an infected cell. Typically the 5' end of the genome has a cap and the 3' end may or may not have a poly (A) tract. *Flavivirus* are usually transmitted by a vector such as an insect, in many cases the insect is a mosquito.

The viral genome of the *Flavivirus* genus is translated as a single polypeptide and is subsequently cleaved into mature proteins. The proteins encoded by the virus typically consist of structural and non-structural proteins. Generally, there are three structural proteins that typically include the envelope protein (E)(amino acids 286-778 of genbank accession number X03700 and SEQ ID NO:2), the core or capsid protein (C)(amino acids 1-121 of genbank accession number X03700), and the pre-membrane protein (preM)(amino acids 122-285 of genbank accession number X03700)(Hahn *et al.*, 1987). The envelope protein is approximately 493 amino acids with an approximate molecular weight of 50 kDa and is often glycosylated. The envelop protein typically contains twelve conserved cysteine residues which form six disulfide

bridges. The core protein is approximately 13 kDa and is rich in arginine and lysine residues. The pre-membrane protein is approximately 10 kDa and is cleaved during or after release of the virus from infected cells. A cleavage product of the prM protein remains associated with the virion and is approximately 8 kDa and is termed the membrane protein (M). Typically, it is the carboxy terminus of prM that remains associated with the virus particle as the M protein.

The computer databases contain a few entries representative of the Yellow Fever virus genome, which is based on three West African strains and a Trinidad strain. Examples of Genbank entries for representative Yellow Fever virus strains may be found under the following accession numbers: 17D-204 (accession No. X15061), 17D-213 (accession No. U17067), 17DD (accession No. U17066), 17D (accession No. X03700). French viscerotropic virus (accession No. U21056), and French neurotropic virus (accession No. U21055), each of which is incorporated herein by reference. Various other strains or isolates are available in the Genbank, ATCC, or other databases/depositories.

Various members of the *Flaviviridae* family are available through the American Type Culture Collection (Manassas Va.) under the following ATCC numbers: Dengue type 1 (VR-71), Ilheus (VR-73), Japanese encephalitis (VR-74), Murray valley encephalitis (VR-77), Ntaya (VR-78), St Louis encephalitis (VR-80), Uganda S (VR-81), West Nile (VR-82), Zika (VR-84), Dengue type 4 (VR-217), Dengue type 2 (VR-222), Japanese encephalitis (VR-343), Dengue type 1 (VR-344), Dengue type 2 (VR-345), Edge hill (VR-377), Entebbe bat (VR-378), Kokobera (VR-379), Stratford (VR-380), Tembusu (VR-381), Dakar bat (VR-382), Ntaya (VR-78), Banzi (VR-414), Modoc (VR-415), Rio Bravo virus (VR-416), Cowbone ridge (VR-417), Bukalasa (VR-418), Montana myotis leukoencephalitis (VR-537), Bussuquara (VR-557), Sepik (VR-906), Cowbone ridge (VR-1253), Dengue type 2 (VR-1255), Dengue type 3 (VR-1256), Dengue type 4 (VR-1257), Ilheus (VR-1258), Rio Bravo virus (VR-1263), St. Louis encephalitis (VR-1265), West Nile (VR-1267), Dengue type 4 (VR-1490), West Nile (VR-1507), and West Nile (VR-1510), each of which is incorporated herein by reference.

A. Yellow Fever virus

Yellow Fever, as described by the World Health Organization (WHO), is a viral disease that has caused large epidemics in Africa and the Americas. Yellow Fever virus infection causes a wide spectrum of disease, from mild symptoms to severe illness and death. Although an effective vaccine is available, the number of people infected over the last two decades has increased and Yellow Fever is now a serious public health issue again.

The Yellow Fever virus belongs to the *Flavivirus* genus. In Africa there are five distinct genetic types (called genotypes) associated with East, Central and West Africa (Mutebi *et al.*, 2001). Also, South America has at least two different genotypes.

The virus remains silent in the body during an incubation period of three to six days. There are then two disease phases. While some infections have no symptoms whatsoever, the first, "acute", phase is normally characterized by fever, muscle pain (with prominent backache), headache, shivers, loss of appetite, nausea and/or vomiting. Often, the high fever is paradoxically associated with a slow pulse. After three to four days most patients improve and their symptoms disappear.

However, 15% enter a "toxic phase" within 24 hours. Fever reappears and several body systems are affected. The patient rapidly develops jaundice and complains of abdominal pain with vomiting. Bleeding can occur from the mouth, nose, eyes and/or stomach. Once this happens, blood appears in the vomit and feces. Kidney function deteriorates; this can range from abnormal protein levels in the urine (albuminuria) to complete kidney failure with no urine production (anuria). Up to half of the patients in the "toxic phase" die within 10-14 days. The remainder recover without significant organ damage.

Yellow Fever is difficult to recognize, especially during the early stages. It can easily be confused with malaria, typhoid, rickettsial diseases, hemorrhagic viral fevers (e.g. Lassa), arboviral infections (e.g. dengue), leptospirosis, viral hepatitis and poisoning (e.g. carbon tetrachloride). A laboratory analysis is required to confirm a suspected case. Blood tests (serology assays) can detect Yellow Fever antibodies that are produced in response to the infection. Several other techniques are used to identify the virus itself in blood specimens or liver tissue collected after death.

B. Flaviviral Nucleic Acid Compositions

The present invention concerns *flaviviruses* that are advantageous in the study and treatment of a variety of diseases. It concerns *flaviviruses*, particularly Yellow Fever viruses, that have been either derived from serial passage in a model host organism, such as a hamster, or constructed with one or more nucleotide alterations compared to wild-type or vaccine strains, such that the virus has desirable properties for use against viral infection, while being less likely to revert to a virulent phenotype. The teachings described herein provide various methods, by way of example, of implementing methods and compositions of the invention. They provide background for generating altered or mutant viruses through the use of propagation in a model host, as well as the genetic engineering of viruses to reduce the probability of reversion to a virulent phenotype. Genetic engineering may include various known methods of manipulating nucleic acid to produce a desired nucleic acid sequence (see Sambrook *et al.*, 1989)

In certain embodiments, the present invention concerns generating a Yellow Fever virus with an altered phenotype, for example a virus that is more virulent than a parental form of the virus; an example of a parental strain is the Asibi strain of Yellow Fever virus. In other embodiments, the present invention concerns analyzing the resultant more virulent virus(es) and using this information to engineer an improved strain of virus for vaccination. This improved strain of virus may be used in combination with proteinaceous compositions as part of a pharmaceutically acceptable formulation. Compositions of the invention may be used as a vaccine to vaccinate an organism against Yellow Fever virus infection

C. Nucleic Acid Molecules

1. Polynucleotides Encoding Native Proteins or Modified Proteins

The present invention concerns polynucleotides, isolatable from cells or virions, that are capable of expressing all or part of a protein, polypeptide, and/or virus. In some embodiments of the invention, it concerns a viral genome that has been specifically mutated to generate a virus with a virulent phenotype or an improved characteristic or property, *e.g.*, a reduced probability of reversion. The polynucleotides may encode a peptide, polypeptide, and/or virus containing all or part

of a viral amino acid sequence or they may be engineered so they do not encode such a viral polypeptide or encode a viral polypeptide having at least one function or activity reduced, diminished, or absent. The polynucleotides may comprise a chimeric virus, a virus derived from genetic material of two separate viruses.

As used herein, the term "nucleic acid segment" refers to a nucleic acid molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a nucleic acid segment encoding a polypeptide refers to a nucleic acid segment that contains wild-type, polymorphic, or mutant polypeptide-coding sequences yet is isolated away from, or purified free from, total mammalian or human genomic DNA. Included within the term "nucleic acid segment" are a polynucleotide or polynucleotides, nucleic acid segments smaller than a polynucleotide, and recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

As used in this application, the term "*flavivirus* polynucleotide or nucleic acid" refers to a nucleic acid molecule encoding a *flavivirus* or a flaviviral polypeptide that has been isolated free of total genomic nucleic acid. Similarly, a "Yellow Fever virus polynucleotide or nucleic acid" refers to a nucleic acid molecule encoding a Yellow Fever virus or a Yellow Fever viral polypeptide that has been isolated free of total genomic nucleic acid. A "*flavivirus* genome" or a "Yellow Fever virus genome" refers to a nucleic acid molecule that can be provided to a host cell to yield a viral particle, in the presence or absence of a helper virus. The genome may or may have not been genetically altered as compared to wild-type virus.

The term "cDNA" is intended to refer to DNA prepared using messenger RNA (mRNA) or RNA encoding polypeptides as a template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic, non- or partially-processed RNA template, is that the cDNA primarily contains coding sequences of the corresponding protein.

It also is contemplated that a particular polypeptide from a given species may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein (see Table 1).

Similarly, a polynucleotide comprising an isolated or purified wild-type or mutant gene refers to a nucleic acid segment including wild-type or mutant polypeptide coding sequences and, in certain aspects, regulatory sequences, isolated

substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide, or peptide-encoding unit (including any sequences required for proper transcription, post-translational modification, or localization). As will be understood by those in the art, this functional term includes genomic sequences, positive strand RNA, cDNA sequences, and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a native or modified polypeptide may contain a contiguous nucleic acid sequence encoding all or a portion of such a polypeptide of the following lengths: 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1095, 1100, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 9000, 10000, 10,862, 11,000 or more nucleotides, nucleosides, or base pairs.

In particular embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode a wild-type or mutant *flavivirus*, in particular Yellow Fever virus, polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to a native polypeptide. Thus, an isolated nucleic acid segment or vector containing a nucleic acid segment may encode, for example, an envelope protein. The term "recombinant" may be used in conjunction with a polypeptide or the name of a specific polypeptide, and this generally refers to a polypeptide produced from a nucleic acid molecule that has been manipulated *in vitro*, *in situ* or that is the replicated product of such a molecule.

In other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode a polypeptide or peptide that includes within its amino acid sequence a contiguous

amino acid sequence in accordance with, or essentially corresponding to the polypeptide.

The nucleic acid segments used in the present invention, regardless of the length of the coding sequence itself, may be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

It is contemplated that the nucleic acid constructs of the present invention may encode full-length polypeptide from any source or encode a truncated version of the polypeptide, for example a truncated Yellow Fever virus polypeptide, such that the transcript of the coding region represents the truncated version. The truncated transcript may then be translated into a truncated protein. Alternatively, a nucleic acid sequence may encode a full-length polypeptide sequence with additional heterologous coding sequences, for example to allow for purification of the polypeptide, transport, secretion, post-translational modification, protease cleavage or for therapeutic benefits such as targeting, antigenicity or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a polypeptide that is not the same as the modified polypeptide.

In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to the a particular gene or segment of a viral genome, such as the envelope protein gene. A nucleic acid construct may be at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 15,000, 20,000, 30,000, 50,000, 100,000, 250,000, 500,000, 750,000, to at least 1,000,000 nucleotides in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to those of ordinary skill in the art. It will be readily

understood that "intermediate lengths" and "intermediate ranges," as used herein, means any length or range including or between the quoted values (*i.e.*, all integers including and between such values).

The nucleic acid segments used in the present invention encompass biologically functional equivalent modified polypeptides and peptides, for example, a modified envelope protein. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by a human may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements in reversion frequency of a virus, in antigenicity of a protein, or in the efficacy of any treatment or vaccine involving the protein or virus.

In certain embodiments, the invention concerns isolated nucleic acids, nucleic acid segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence from that shown in SEQ ID NO:1, or any other sequence incorporated by reference. Such sequences, however, may be mutated to yield a virus that is altered with respect to a wild-type or a vaccine strain of a virus, *e.g.*, Yellow Fever virus or its vaccine derivatives.

It also will be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:1, 2, 3, 4 or any other sequence incorporated by reference. Recombinant vectors and isolated nucleic acid segments may therefore variously include the Yellow Fever virus-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region or codons, or they may encode larger polypeptides that nevertheless include viral-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acid sequences.

The nucleic acid segments of the present invention encompass biologically functional equivalent Yellow Fever virus proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded.

Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged or their representative codons. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the virus resulting in a reduced probability of reversion to a virulent phenotype.

2. Mutagenesis of Flaviviral Polynucleotides

Where employed, mutagenesis will be accomplished by a variety of standard, mutagenic procedures, including passaging virus through cell lines or animals, and standard molecular biological techniques, for exemplary methods see Tech *et al.* 2001 and Sambrook *et al.,* 1989. Mutation is the process whereby changes occur in the quantity or structure of a nucleic acid, a polypeptide, or an organism. Mutation can involve modification of a single nucleotide, the nucleotide sequence of a single gene, blocks of genes or whole chromosomes or genomes. Changes in single genes may be the consequence of point mutations which involve the removal, addition or substitution of a single nucleotide base within a nucleic acid sequence, or they may be the consequence of changes involving the insertion or deletion of large numbers of nucleotides.

Mutations may be induced following exposure to chemical or physical mutagens. Such mutation-inducing agents include ionizing radiation, ultraviolet light (U.V.) and a diverse array of chemicals such as alkylating agents and polycyclic aromatic hydrocarbons all of which are capable of interacting either directly or indirectly (generally following some metabolic biotransformations) with nucleic acids. The DNA damage induced by such agents may lead to modifications of base sequence when the affected DNA is replicated or repaired and thus to a mutation. Mutation also can be site-directed through the use of particular targeting methods, such as oligo directed site directed mutatgenesis.

a. Random Mutagenesis

i) Insertional Mutagenesis

Insertional mutagenesis is based on the inactivation of a gene via insertion of a known DNA fragment. Because it involves the insertion of some type of DNA fragment, the mutations generated are generally loss-of-function, rather than gain-of-function mutations. However, there are several examples of insertions generating gain-of-function mutations (Oppenheimer et al. 1991). Insertion mutagenesis has been very successful in bacteria and Drosophila (Cooley et al. 1988) and recently has become a powerful tool in corn (Schmidt et al. 1987); Arabidopsis; (Marks et al., 1991; Koncz et al. 1990); and Antirrhinum (Sommer et al. 1990). Insertional mutagenesis may be accomplished using standard molecular biology techniques.

ii) Chemical mutagenesis

Chemical mutagenesis offers certain advantages, such as the ability to find a full range of mutations with degrees of phenotypic severity, and is facile and inexpensive to perform. The majority of chemical carcinogens produce mutations in DNA. Benzo[a]pyrene, N-acetoxy-2-acetyl aminofluorene and aflotoxin B1 cause GC to TA transversions in bacteria and mammalian cells. Benzo[a]pyrene also can produce base substitutions such as AT to TA. N-nitroso compounds produce GC to AT transitions. Alkylation of the O4 position of thymine induced by exposure to n-nitrosoureas results in TA to CG transitions.

iii) Radiation Mutagenesis

Biological molecules are degraded by ionizing radiation. Adsorption of the incident energy leads to the formation of ions and free radicals, and breakage of some covalent bonds. Susceptibility to radiation damage appears quite variable between molecules, and between different crystalline forms of the same molecule. It depends on the total accumulated dose, and also on the dose rate (as once free radicals are present, the molecular damage they cause depends on their natural diffusion rate and thus upon real time). Damage is reduced and controlled by making the sample as cold as possible. Ionizing radiation causes DNA damage, generally proportional to the dose rate.

In the present invention, the term "ionizing radiation" means radiation comprising particles or photons that have sufficient energy or can produce sufficient energy via nuclear interactions to produce ionization (gain or loss of electrons). An exemplary and preferred ionizing radiation is an x-radiation. The amount of ionizing radiation needed in a given cell generally depends upon the nature of that cell and the

nature of the mutation target. Means for determining an effective amount of radiation are well known in the art.

iv) In Vitro Scanning Mutagenesis

Random mutagenesis also may be introduced using error prone PCR (Cadwell and Joyce, 1992). The rate of mutagenesis may be increased by performing PCR in multiple tubes with dilutions of templates.

One particularly useful mutagenesis technique is alanine scanning mutagenesis in which a number of residues are substituted individually with the amino acid alanine so that the effects of losing side-chain interactions can be determined, while minimizing the risk of large-scale perturbations in protein conformation (Cunningham et al., 1989).

In vitro scanning saturation mutagenesis provides a rapid method for obtaining a large amount of structure-function information including: (i) identification of residues that modulate ligand binding specificity, (ii) a better understanding of ligand binding based on the identification of those amino acids that retain activity and those that abolish activity at a given location, (iii) an evaluation of the overall plasticity of an active site or protein subdomain, (iv) identification of amino acid substitutions that result in increased binding.

b. Site-Directed Mutagenesis

Structure-guided site-specific mutagenesis represents a powerful tool for the dissection and engineering of proteins. The technique provides for the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into a selected DNA.

Site-specific mutagenesis uses specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent, unmodified nucleotides. In this way, a primer sequence is provided with sufficient size and complexity to form a stable duplex on both sides of the deletion junction being traversed. A primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Vectors useful in site-directed

mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double-stranded plasmids are also routinely employed in site-directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, one first obtains a single-stranded vector, or melts two strands of a double-stranded vector, which includes within its sequence a DNA sequence encoding the desired protein or genetic element. An oligonucleotide primer bearing the desired mutated sequence, synthetically prepared, is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions. The hybridized product is subjected to DNA polymerizing enzymes such as E. coli polymerase I (Klenow fragment) in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed, wherein one strand encodes the original non-mutated sequence, and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells, such as E. coli cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

Comprehensive information on the functional significance and information content of a given residue of protein can best be obtained by saturation mutagenesis in which all 19 amino acid substitutions are examined. The shortcoming of this approach is that the logistics of multiresidue saturation mutagenesis are daunting (Warren et al., 1996, Brown et al., 1996; Zeng et al., 1996; Burton and Barbas, 1994; Yelton et al., 1995; Jackson et al., 1995; Short et al., 1995; Wong et al., 1996; Hilton et al., 1996). Hundreds, and possibly even thousands, of site specific mutants must be studied. However, improved techniques make production and rapid screening of mutants much more straightforward. See also, U.S. Patents 5,798,208 and 5,830,650, for a description of "walk-through" mutagenesis. Other methods of site-directed mutagenesis are disclosed in U.S. Patents 5,220,007; 5,284,760; 5,354,670; 5,366,878; 5,389,514; 5,635,377; and 5,789,166.

D. Oligonucleotide Probes and Primers

Naturally, the present invention also encompasses nucleic acid segments that are complementary, or essentially complementary, to all or part of the sequence set forth in SEQ ID NO:1, or any other sequence incorporated by reference. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:1, or any other sequence incorporated by reference, under relatively stringent conditions such as those described herein. Such sequences may encode the entire sequence of *flavivirus* genome or functional or non-functional fragments thereof.

Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence in the presence of various nucleic acids. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, 3000 or 3431 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and RNA blots and as primers in nucleic acid amplification reactions.

Suitable hybridization conditions will be well known to those of skill in the art. In certain applications, for example, substitution of amino acids by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low

stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C. Formamide and SDS also may be used to alter the hybridization conditions.

One method of using probes and primers of the present invention is in the search for other viral sequences related to Yellow Fever virus or, more particularly, homologs of the envelope protein or other yellow virus protein sequences. By varying the stringency of hybridization, and the region of the probe, different degrees of homology may be discovered.

Another way of exploiting probes and primers of the present invention is in site-directed, or site-specific, mutagenesis. The technique provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into complementary nucleic acid. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the nucleic acid sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion or mutation junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed

mutagenesis, which eliminates the step of transferring the nucleic acid of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a nucleic acid sequence encoding the desired protein or protein segment, protein segment being any part or fragment of an encoded protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded nucleic acid preparation, taking into account the degree of mismatch when selecting hybridization conditions, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement. There are newer and simpler site-directed mutagenesis techniques that can also be employed for this purpose. These include procedures marketed in kit form that are readily available to one of ordinary skill in the art.

The preparation of sequence variants of the selected nucleic acid using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of nucleic acids may be obtained. For example, recombinant vectors encoding the desired nucleic acid segment may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

E. Proteinaceous Compositions

Embodiments of the invention may include viral particles, including proteins and polypeptides associated with *flavivirus* particles. In various embodiments the viral particles may be produced and/or propagated from an altered nucleic acid encoding a *flavivirus*, in particular a Yellow Fever virus. In certain embodiments the altered nucleic acid encodes a virus with enhanced virulence. In other embodiments the nucleic acid may be engineered to encode a virus with a reduced probability of reverting to a virulent phenotype. As used herein, a "protein" or "polypeptide" refers

to a molecule comprising at least one amino acid residue. In some embodiments, a wild-type version of a protein or polypeptide may be employed, however, in many embodiments of the invention, a viral protein or polypeptide is absent or altered so as to render the virus more useful for the treatment of a subject or patient. The terms described above may be used interchangeably herein. A "modified protein" or "modified polypeptide" refers to a protein or polypeptide whose chemical structure is altered with respect to the wild-type or parental (*i.e.*, a *flavivirus* polynucleotide to be altered, which may be a vaccine strain and not considered wild-type) protein or polypeptide. In some embodiments, a modified protein or polypeptide has at least one modified activity or function (recognizing that proteins or polypeptides may have multiple activities or functions). The modified activity or function may be reduced, diminished, eliminated, enhanced, improved, or altered in some other way (such as specificity or propensity to revert to a virulent phenotype) with respect to that activity or function in a wild-type or vaccine protein or polypeptide. It is specifically contemplated that a modified protein or polypeptide may be altered with respect to one activity or function yet retain wild-type or vaccine activity or function in other respects. All or part of a *flavivirus* encoded protein may be isolated using known recombinant techniques and used as part of proteinaceous composition, *e.g.*, as a peptide vaccine or to generate *flavivirus* specific antibodies.

In certain embodiments the size of a protein or polypeptide may comprise, but is not limited to, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2250, 2500 or greater amino molecule residues, and any range derivable therein.

As used herein, an "amino molecule" refers to any amino acid, amino acid derivative or amino acid mimic as would be known to one of ordinary skill in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule

residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

Accordingly, the term "proteinaceous composition" encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid.

In certain embodiments the proteinaceous composition comprises at least one protein, polypeptide or peptide. In further embodiments the proteinaceous composition comprises a biocompatible protein, polypeptide or peptide. As used herein, the term "biocompatible" refers to a substance that produces no significant untoward effects when applied to, or administered to, a given organism according to the methods and amounts described herein. Such untoward or undesirable effects are those such as significant toxicity or adverse immunological reactions. In preferred embodiments, biocompatible protein, polypeptide or peptide containing compositions will generally be essentially free from toxins, pathogens and harmful immunogens.

Proteinaceous compositions may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteinaceous compounds from natural sources, or the chemical synthesis of proteinaceous materials.

In certain embodiments a proteinaceous compound may be purified. Generally, "purified" will refer to a specific protein, polypeptide, or peptide composition that has been subjected to fractionation to remove various other proteins, polypeptides, or peptides, and which composition substantially retains its activity, as may be assessed, for example, by the protein assays, as would be known to one of ordinary skill in the art for the specific or desired protein, polypeptide or peptide.

1. Variants of Viral Polypeptides

Alteration in the nucleic acids encoding a *flavivirus* may be altered so that the probability of a virus reverting to a virulent phenotype is reduced. Nucleic acid alteration(s) may include the substitution of an amino acid in a vaccine strain with a conservative or non-conservative amino acid, so that multiple mutations are needed to change an amino acid in a vaccine or other virus strain to an amino acid present in a virulent virus.

Amino acid sequence variants of the polypeptides of the present invention can be substitutional, insertional or deletion variants. A mutation in a gene encoding a viral polypeptide may affect 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more non-contiguous or contiguous amino acids of the polypeptide, as compared to wild-type.

Deletion variants lack one or more residues of the parental, native or wild-type protein. Individual residues can be deleted or all or part of a domain (such as a catalytic or binding domain) can be deleted. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply one or more residues. Terminal additions, called fusion proteins, may also be generated.

In certain embodiments, substitutions will be made so that multiple mutations in a codon will be necessary to encode for a amino acid that is associated with increased virulence. Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, with or without the loss of other functions or properties. Substitutions may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or

glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Alternatively, substitutions may be non-conservative such that a function or activity of the polypeptide is affected or is not affected. Non-conservative changes typically involve substituting a residue with one that is chemically dissimilar, such as a polar or charged amino acid for a nonpolar or uncharged amino acid, and vice versa.

The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 1, below).

TABLE 1

Codon Table

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The

addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, cellular receptors or binding sites on target or immune effector cells. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity and still result in a vaccine with a reduced probability of reversion to a virulent form of *flavivirus*. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

II. METHODS OF DETECTION

In various embodiments, the detection of *flavivirus*, in particular Yellow Fever virus, may be used to identify infection by a virulent form of the virus or to confirm the identity of a particular vaccine or strain. Detection methods may use the antigenic properties of a virus particle or the properties of the nucleic acid component of the virus to identify and/or detect the presence of a virus.

A. Nucleic Acid Detection

In addition to their use in directing the expression of *flavivirus* proteins, polypeptides and/or peptides, the nucleic acid sequences disclosed herein have a variety of other uses. For example, they have utility as probes or primers for embodiments involving nucleic acid hybridization or amplification. They may be used in diagnostic or screening methods of the present invention. Detection of nucleic acids encoding *flavivirus* or *flavivirus* polypeptide modulators are encompassed by the invention.

1. Hybridization

The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over contiguous stretches

greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific nucleic acids or for detecting specific RNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator

substrates are known that can be employed to provide a detection means that is visibly or spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding nucleic acids, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art. After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patents 5,843,663, 5,900,481 and 5,919,626, each of which is incorporated herein by reference. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,481, 5,849,486 and 5,851,772, also incorporated herein by reference.

2. Amplification of Nucleic Acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues, viral isolates, blood or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA, viral RNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be

provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to SEQ ID NO:1, or any other sequence incorporated by reference, or any other segment thereof corresponding to a nucleic acid sequence are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals (Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988, each of which is incorporated herein by reference in their entirety. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCR™ and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366,

5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety. Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

3. Detection of Nucleic Acids

Following any amplification, it may be desirable to separate the amplification product from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989).

In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and

visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under the appropriate excitatory spectra.

In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art (see Sambrook *et al.*, 1989). One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

Other methods of nucleic acid detection that may be used in the practice of the instant invention are disclosed in U.S. Patents 5,840,873, 5,843,640, 5,843,651, 5,846,708, 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

4. Other Assays

Other methods for genetic screening may be used within the scope of the present invention, for example, to detect mutations in genomic RNA, cDNA and/or RNA samples. Methods used to detect point mutations include denaturing gradient gel electrophoresis ("DGGE"), restriction fragment length polymorphism analysis ("RFLP"), chemical or enzymatic cleavage methods, direct sequencing of target regions amplified by PCR™ (see above), single-strand conformation polymorphism analysis ("SSCP") and other methods well known in the art.

One method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA or RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule.

This definition thus includes mismatches due to insertion/deletion mutations, as well as single or multiple base point mutations.

U.S. Patent 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

Other investigators have described the use of RNase I in mismatch assays. The use of RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is reported to cleave three out of four known mismatches. Others have described using the MutS protein or other DNA-repair enzymes for detection of single-base mismatches.

Alternative methods for detection of deletion, insertion or substitution mutations that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,483, 5,851,770, 5,866,337, 5,925,525 and 5,928,870, each of which is incorporated herein by reference in its entirety.

B. Protein Detection

In various embodiments, *Flavivirus*, in particular Yellow Fever virus, may be detected by using polyclonal or monoclonal antibodies in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to *flavivirus*-related antigen epitopes. For general methodologies regarding antibody production and utilization see Harlow and Lane, 1988; and Sambrook *et al.*, 1989, each of which is incorporated herein by reference.

III. PHARMACEUTICAL FORMULATIONS

In various embodiments of the present invention, a method of treatment or prophylaxis for a viral infection is contemplated. Examples of viral infection contemplated for treatment include Yellow Fever virus, Japanese encephalitis virus, Dengue fever virus, West Nile virus, hepatitis C virus, St. Louis encephalitis virus,

and other members of the *flavivirus* genus described herein may be treated. Vaccines of the invention may be suitable to induce an immune response against a *flavivirus*, Yellow Fever virus or a derivative thereof. See U.S. Patent Nos. 6,372,221, 6,337,073, 6,254,873, 6,184,024, 6,171,854, 5,744,141, 5,744,140, 5,736,148, 4,810,492, and 4,500,512, each incorporated herein by reference, for exemplary methods and compositions related to *flavivirus* and there use in vaccines.

An exemplary vaccine composition may include a Yellow Fever virus with a viral genome with at least one of the following alterations: a) an alteration in the nucleic acid sequence encoding amino acid 323 of an/the envelope protein, wherein the first alteration requires more than one nucleotide change to encode an arginine; b) an alteration in the nucleic acid sequence encoding amino acid 27 of an/the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a histidine; etc., c) an alteration in the nucleic acid sequence encoding amino acid 28 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a glycine; d) an alteration in the nucleic acid sequence encoding amino acid 155 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a alanine; e) an alteration in the nucleic acid sequence encoding amino acid 331 of the envelope protein, wherein the second alteration requires more than one nucleotide change to encode a arginine; f) an alteration in the nucleic acid sequence encoding amino acid 48 of the NS2A protein, wherein the second alteration requires more than one nucleotide change to encode a alanine; or g) an alteration in the nucleic acid sequence encoding amino acid 98 of the NS4B protein, wherein the second alteration requires more than one nucleotide change to encode a isoleucine. In other embodiments the viral genome may include one, two, three, four, five, six, or seven of the above alterations. In yet other embodiments, the vaccine compositions described herein may be used in methods of vaccination that include administering the vaccine compositions to a subject in need of vaccination. Each of these alteration may be used in conjunction with any other combination of alteration. Such that any one alteration may be used in combination with one, two, three, four, five, or six of the other alterations described herein.

An effective amount of the pharmaceutical composition, generally, is defined as that amount sufficient to detectably and repeatedly ameliorate, reduce, minimize or

limit the extent of the infection, disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

Pharmaceutical compositions of the present invention comprise an effective amount of one or more attenuated virus of the *Flaviviridae* family with a mutant or altered viral genome and/or additional agent(s) dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that contains at least one attenuated virus of the *Flaviviridae* family with a mutant or altered viral genome and/or additional agent(s) dissolved or dispersed in a pharmaceutically acceptable carrier will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 1990, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

The attenuated virus of the invention may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or

ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine.

The present invention contemplates vaccines for use in both active and passive immunization, in certain embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from attenuated virus of the *Flaviviridae* family with a mutant or altered viral genome, prepared in a manner disclosed herein. In various embodiments, an antigenic material may be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

Typically, vaccines are prepared as injectables. Either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, etc), lipids (e.g., triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines. Additionally, iscom, a supramolecular spherical structure, may be used for parenteral and mucosal vaccination (Morein et al., 1998).

Sterile injectable solutions may be prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation

are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

Various methods of achieving adjuvant effect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed.

Adjuvants that may be used in the practice of the invention include, but are not limited to Adjumer™, Adju-Phos, Algal Glucan, Algammulin, Alhydrogel, Antigen Formulation, Avridine®, BAY R1005, Calcitriol, Calcium Phosphate Gel, Cholera holotoxin (CT), Cholera toxin B subunit (CTB), Cholera toxin A1-subunit-Protein A D-fragment fusion protein, CRL1005, Cytokine-containing Liposome, Dimethyldioctadecylammonium bromide, Dehydroepiandrosterone; Dimyristoyl phosphatidylcholine; 1,2-dimyristoyl-sn-3-phosphatidylcholine, Dimyristoyl phosphatidylglycerol, Deoxycholic Acid Sodium Salt; Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, Gamma Inulin, Gerbu Adjuvant, GM-CSF, N-acetylglucosaminyl-(β1-4)-N-acetylmuramyl-L-alanyl-D-isoglutamine, Imiquimod, ImmTherTM, Interferon-γ, Interleukin-1β, Interleukin-2, Interleukin-7, Interleukin-12, ISCOMTM, Iscoprep 7.0.3.TM, Liposome, Loxoribine, LT-OA or LT Oral Adjuvant, MF59, MONTANIDE ISA 51, MONTANIDE ISA 720, MPLTM, MTP-

PE, MTP-PE Liposome, Murametide, Murapalmitine, D-Murapalmitine, NAGO, Non-Ionic Surfactant Vesicle, Pleuran, lactic acid polymer,glycolic acid polymer, Pluronic L121, Polymethyl methacrylate, PODDSTM, Poly rA:Poly rU, Polysorbate 80, Protein Cochleate, QS-21, Quil-A, Rehydragel HPA, Rehydragel LV, S-28463, SAF-1, Sclavo peptide, Sendai Proteoliposome, Sendai-containing Lipid Matrix, Span 85, Specol, Squalane, Squalene, Stearyl Tyrosine, Theramide™, Threonyl-MDP, Ty Particle, or Walter Reed Liposome.

Any of the conventional methods for administration of a vaccine are applicable. These include, but are not limited to oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. Vaccines of the invention may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include, in some cases, oral formulations. In other embodiments, one may use eye drops, nasal solutions or sprays, aerosols or inhalants in the present invention. Such compositions are generally designed to be compatible with the target tissue type. In a non-limiting example, nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, in preferred embodiments the aqueous nasal solutions usually are isotonic or slightly buffered to maintain a pH of about 5.5 to about 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, drugs, or appropriate drug stabilizers, if required, may be included in the formulation. For example, various commercial nasal preparations are known and include drugs such as antibiotics or antihistamines.

In certain embodiments, the attenuated virus of the invention is prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (e.g., hard or soft shelled gelatin capsules), sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with the food of the diet. Preferred carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention,

the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

In certain preferred embodiments, an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc.; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. Oral formulations may contain about 10 to about 95% of active ingredient, preferably about 25 to about 70%.

In certain embodiments, vaccines may comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more of antigen or

total protein per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescents, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

"Unit dose" is defined as a discrete amount of a therapeutic composition dispersed in a suitable carrier. For example, in accordance with the present methods, viral doses include a particular number of viral or plaque forming units (pfu). For embodiments involving virus, particular unit doses include 10^1, $10^2 10^3$, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14} or 10^{15} pfu or viral particles (vp).

In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, a unit

dose could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein.

In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

IV. SCREENING ASSAYS

The present invention also contemplates the screening of compounds for various abilities to interact and/or affect *flavivirus*, in particular Yellow Fever virus, function and/or infectivity. Particularly preferred compounds will be those useful in inhibiting viral infection of cells, tissues, or organs. In the screening assays of the present invention, the candidate substance may first be screened for basic biochemical activity - *e.g.*, binding to Yellow Fever virus - and then tested for its ability to modulate activity or infectivity, at the cellular, tissue or whole animal level.

A. Assay Formats

The present invention provides methods of screening for modulators of yellow fever virus infectivity. In one embodiment, the present invention is directed to a method of:

(i) providing a Yellow Fever virus;

(ii) contacting the Yellow Fever virus with a candidate substance; and

(iii) determining the binding of the candidate substance to the Yellow Fever virus.

In yet another embodiment, the assay looks not at binding, but at viral infectivity. Such methods would comprise, for example:

(i) providing a cell that is susceptible to Yellow Fever virus infection;
(ii) contacting the virus with the candidate substance; and
(iii) determining the effect of the candidate substance on infectivity of Yellow Fever virus.

In still yet other embodiments, one would look at the effect of a candidate substance on the activity of Yellow Fever virus. This may involve looking at any of a number of characteristics, including Yellow Fever virus gene expression. An exemplary assay may include the detection of Yellow Fever virus nucleic acid by PCR.

B. Candidate Substances

As used herein, the term "candidate substance" refers to any molecule that may potentially modulate Yellow Fever virus infectivity. The candidate substance may be a protein or fragment thereof, a small molecule inhibitor, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to compounds which interact naturally with Yellow Fever virus or its family members. Creating and examining the action of such molecules is known as "rational drug design," and include making predictions relating to the structure of target molecules.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for a molecule like yellow virus envelope protein, and then design a molecule for its ability to interact with the envelope protein. Alternatively, one could design a partially functional fragment of an envelope protein (binding but no activity),

thereby creating a competitive inhibitor. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

Candidate compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors of a steroid hormone receptor repressor.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be

considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1:

METHODS

Animals:

The animals used in these studies were 3-4 week-old, female, Syrian golden hamsters (*Mesocricetus auratus*) from Harlan Sprague Dawley.

Hamster passages:

A single hamster was inoculated intraperitoneally (i.p.) with Asibi virus. At 3 days post infection (dpi), the liver was harvested and homogenized in PBS. After freezing at -70° overnight, 100µl of the liver homogenate was inoculated i.p. into a naïve hamster and is termed liver-to-liver passage. This process was repeated 6 times to generate the viscerotropic Asibi/hamster p7 virus.

Titration of viruses:

Serum was obtained by saphenous vein bleed each day for 6 days following i.p. inoculation with either wild-type Asibi/hamster p0 or viscerotropic Asibi/hamster p7. Virus titer in the serum was determined by tissue culture infectious dose 50% (TCID50) in Vero cells.

Morbidity and mortality:

Hamsters were inoculated i.p. with Asibi/hamster p0 or Asibi/hamster p7 virus and observed for signs of illness for 14 days. Signs of illness included: ruffled fur, lethargy, hunched posture, and paralysis. Some animals found to be completely moribund were euthanized to collect organ samples. These animals are not included in the survival curve.

Histopathology:

Liver and spleen were harvested 5 and 6 dpi for histological examination. The tissues were fixed in 10% buffered formalin for 48 hours and then transferred to 70% ethanol for storage. The tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin by the core facility (UTMB).

Sequence analysis:

Viral RNA was isolated using the QIamp viral RNA mini kit (Qiagen). The genome was amplified by RT-PCR with YF virus specific primers. Fragments were cloned into either pGEM-T (Promega) or pCR (Invitrogen) vector and amplified in DH5α competent cells. A consensus sequence was taken from 3 or more clones sequenced in both directions. Automated sequencing was performed in the UTMB core laboratory. Sequence analysis was performed using the Vector NTI program (InforMax).

EXAMPLE 2

Passage of wild-type YF virus Asibi in hamsters:

Wild-type, non-hamster passaged Asibi (p0) virus causes a mild and transient viremia with no outward signs of illness in sub-adult hamsters. The 7th hamster passage (Asibi/hamster p7) virus was found to be highly viscerotropic in hamsters and caused severe illness and death in 100% of sub-adult hamsters.

Morbidity and mortality:

Sub-adult hamsters were inoculated with either the parental Asibi/hamster p0 or the hamster-viscerotropic Asibi/hamster p7 virus and observed for 14 days. Hamsters inoculated with Asibi/hamster p0 virus showed no outward signs of illness, and all animals survived. In contrast all 7 hamsters inoculated with Asibi/hamster p7 developed outward signs of illness including ruffled fur, lethargy, and hunched posture and died within 2 days of onset of clinical signs of disease. Signs of illness appeared as early as 2 dpi, and all animals succumbed to illness by 8 dpi. The survival of these animals is summarized in FIG. 1.

Viremia:

Hamsters inoculated with Asibi/hamster p7 virus developed a robust viremia that peaked at 3dpi (FIG. 2), as shown with other strains of YF virus by Tesh et al. (2001). Only a modest viremia was detected in hamsters inoculated with Asibi/hamster p0, and no viremia was detected in 2 of 5 animals (FIG. 2).

Histopathology

Spleen and liver were harvested on 5-6 dpi (at a time determined by Xiao et al (2001) to be the peak of histopathologic changes). Samples from 5 animals (A-E)

inoculated with either Asibi/hamster p0 or Asibi/hamster p7 were paraffin embedded and stained with hematoxylin and eosin for microscopic evaluation.

Liver-

The livers of hamsters inoculated with Asibi/hamster p0 showed no significant changes on either day 5 or 6 pi when compared with mock-infected animals (FIG. 3A and 3B). However, the livers of hamsters inoculated with Asibi/hamster p7 showed significant pathologic changes including microvesicular steatosis, moderate to severe inflammation, and mild to moderate hepatic necrosis (FIG. 3C). Hamster A was sacrificed on day 5 pi due to extreme illness. The remaining four hamsters were sacrificed on day 6 pi when they were beginning to show clinical signs of illness. The liver of hamster A had the most pronounced steatosis (98%) and severe hepatic necrosis with only mild/moderate inflammation. The steatosis in the other hamsters involved 50-95% of the liver, with an average of 77% (FIG. 4A). The results of these studies are summarized in FIG. 4.

Spleen-

The spleens of 4 out of 5 hamsters inoculated with Asibi/hamster p0 were characterized by marked lymphoid hyperplasia and moderate to severe white pulp depletion, necrosis, and splenic macrophage hyperplasia (FIG. 5 and 6). The spleen from hamster E showed no abnormalities. There was no lymphoid hyperplasia in any of the spleens from hamsters inoculated with Asibi/hamster p7; however, there was severe splenic macrophage hyperplasia and necrosis. There was also moderate to severe white pulp depletion (FIG. 5 and 6).

EXAMPLE 3

Nucleotide and deduced amino acid changes of Asibi/hamster p7 virus

The complete genomic sequence of the Asibi/hamster p7 virus was determined and compared with that of the published Asibi sequence (Hahn *et al.*, 1987) identifying 23 nucleotide changes. Regions that contained nucleotide changes were amplified from the parental Asibi/hamster p0 virus and sequenced for confirmation. The parental Asibi strain used in these studies was obtained from the World Reference Center. This virus differed from the published sequence for Asibi (Hahn *et al.*, 1987) at genomic nucleotide positions 2193, 2355, 2704, 3817, 3925, 5926, 6013,

6829, and 7319 reducing the number of nucleotide changes between the parental and hamster-passaged viruses from 23 to 14. These 14 nucleotide changes encoded 7 amino acid substitutions (Table 2).

Table 2: Summary of the nucleotide and deduced amino acid changes between Asibi/hamster p0 virus and Asibi/hamster p7 virus.

Nucleotide	Asibi p0	Asibi p7	amino acid	Asibi p0	Asibi p7
802	A	G			
887[b]	C	U			
1000[b]	G	A			
1054[b]	A	C	E27	Q	H
1056	A	G	E28	D	G
1437[b]	A	C	E155	D	A
1941	A	G	E323	K	R
1965[a,b]	A	G	E331	K	R
2779	U	C			
3274[a,b]	G	A			
3821[b,c]	A	G	NS2A48	T	A
4864[a,b]	G	A			
7178[b,c]	G	A	NS4B98	V	I
8917	C	U			

[a] Nucleotide substitutions shared with 17D virus
[b] Nucleotide substitutions shared with Asibi/HeLa p6 virus
[c] Nucleotide substitutions shared with FNV virus

The nucleotide and amino acid substitutions in the Asibi/hamster p7 virus were not evenly distributed throughout the genome (Table 3). No nucleotide changes were identified in the 5' or 3' NCR of the Asibi/hamster p7 virus. There were 8 nucleotide substitutions found within the structural protein genes (2 in the M protein gene and 6 in the E protein gene), and the remaining 6 nucleotide changes were located within the non-structural protein genes (2 in NS1; 1 each in NS2A, NS3, NS4B and NS5). No nucleotide changes were identified within the C, prM, NS2B, NS4A, and 2K protein genes or within the 5' or 3' non-coding regions (NCR). Two amino acid substitutions were located in the non-structural proteins at positions NS2A48 (T to A), and NS4B98 (V to I); however, the majority of the amino acid changes were located in the E protein: E27 (Q to H), E28 (D to G), E155 (D to A), E323 (K to R), E331 (K to R). Only certain regions of the genome can tolerate

mutation; therefore, viable viruses accumulate mutations only within these regions despite strong selective pressures. Many of the nucleotide and amino acid changes identified in the Asibi/hamster p7 virus are common to other derivatives of Asibi (17D and Asibi/HeLa p6), the vaccine strain FNV, and/or wild-type YF viruses. Only 4 nucleotide changes appear to be unique to the Asibi/hamster p7 virus (Table 2) and these encode 1 amino acid substitution at E323 (K to R). A genbank search for amino acids common to those found in Asibi/hamster p7 virus revealed only 3 YF isolates from the East and Central African genotype with a glycine residue at position E28 (Ethiopia 60A and 60B, and CAR 80) (Mutebi *et al.*, 2001). All YF virus sequences in genbank had lysine at residue E 323 where Asibi/hamster p7 had an arginine residue.

Table 3: Distribution of nucleotide and amino acid changes throughout the genome of the Asibi/hamster p7 virus

Region	Length	total ntd changes	% ntd changes	Total aa changes	% aa changes
5'NCR	119	0	*	0	*
C	362	0	*	0	*
PrM	267	0	*	0	*
M	225	2	0.8	0	*
E	1479	6	0.4	5	1.0
NS1	1227	2	0.2	0	*
NS2A	501	1	0.2	1	0.6
NS2B	390	0	*	0	*
NS3	1869	1	0.1	0	*
NS4A	378	0	*	0	*
2K	66	0	*	0	*
NS4B	750	1	0.1	1	0.4
NS5	2715	1	0.04	0	*
3'NCR	511	0	*	0	*

Seven of the 14 nucleotide changes encode amino acid substitutions, and 5 of these are located in the E protein at amino acid positions: E27 (Q to H), E28 (D to G), E155 (D to A), E323 (K to R), E331 (K to R). The location of these amino acid changes has been modeled onto the TBE virus E protein crystal structure (FIG. 7) to investigate the potential interactions of the amino acid substitutions. E27, E28, and E155 are located in domain I with E27 and E28 adjacent to one another and E155 spatially distinct. The other 2 changes E323 and E331 are located relatively close together in domain III. There are no amino acid substitutions within domain II or the stem-anchor region.

EXAMPLE 4

Intracerebral inoculation with Asibi P0 or Asibi P7

A characteristic of viscerotropic strains of wild-type YF virus is that inoculation into the brains of monkeys results in death by viscerotropism (*i.e.*, liver disease) while inoculation of other strains into the brains of monkeys results in death by neurotropism (*i.e.*, disease in the brain). Hamsters were tested for the disease pattern following intracerebral inoculation into the brain of Asibi p0, Asibi p7 and the

French neurotropic vaccine (FNV). As shown in Table 4, Asibi p7 virus killed the animals by viscerotropism while Asibi p0 and FNV killed the hamsters by neurotropism. These results are consistent with previous studies showing that non-human primates inoculated i.c. with viscerotropic strains of YF virus develop viscerotropic disease rather than neurotrophic disease. This provides more evidence that Asibi p7 is a model for viscerotropic disease in primates.

Table 4: Inoculation of hamsters with Asibi P0 v. Asibi P7

	FNV	**Asibi P0**	**Asibi P7**
Type of infection	Neurotropic	Neurotropric	Viscerotropic
Illness/death	4-5 dpi	5-6 dpi	5-9 dpi

EXAMPLE 5

Asibi Hamster Passage 6 Virus

Asibi Hamster passage 6 virus, *i.e.*, one passage before hamster passage 7, was characterized.

The peak viremia on day 3 post infection of Asibi Hamster passage 6 virus was very similar to non-hamster passaged Asibi virus (Asibi p0 is $10^{4.5}$ $TCID_{50}$/ml while Asibi p6 is $10^{3.0}$ $TCID_{50}$/ml) and is much less than Asibi Hamster passage 7 virus ($10^{7.5}$ $TCID_{50}$/ml) (FIG. 8A). Thus, nucleotide/amino acid changes accumulated between Asibi hamster p6 to p7 result in a greatly increased viremia.

Mortality of Asibi p6 virus (36% hamsters) was in-between Asibi p0 (0% hamsters) and Asibi p7 (100% hamsters) (FIG. 8B). Thus, some nucleotide/amino acid changes accumulated during six passages in hamsters result in a virus that is more lethal than the parent virus but not as lethal as Asibi p7 virus.

There are 7 amino acid substitutions found in Asibi hamster p7 virus compared to Asibi p0 virus. Determination of the nucleotide sequence of the genome of Asibi hamster p6 virus revealed that Asibi p6 virus is identical to Asibi p7 virus except for the amino acid substitution at E-28 where Asibi p6 is the same as Asibi p0 (Table 5). This indicates that one or more of the six substitutions found in Asibi hamster p6 virus cause the increased lethality of Asibi p6 virus (0 vs. 36%), while E-28 is responsible for the increased viremia of Asibi p7 virus ($10^{3\text{-}4.5}$ vs $10^{7.5}$ $TCID_{50}$/ml). Note NS4B-98 has an isoleucine in Asibi p7 and probably Asibi p6.

Table 5: Amino acid changes among p0, p6, and p7

Position	Asibi P0	Asibi P6	Asibi P7
E27	Q	H	H
E28	D	D	G
E155	D	A	A
E323	K	R	R
E331	K	R	R
NS2A48	T	A	A
NS4B98	V		I

* * * * * * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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